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Interleukin-34 Stimulates Gut Fibroblasts to Produce Collagen Synthesis

Eleonora Franzè,[a] Vincenzo Dinallo,[a] Federica Laudisi,[a] Antonio Di Grazia,[a] Davide Di Fusco,[a] Alfredo Colantoni,[a] Angela Ortenzi,[a] Paolo Giuffrida,[b] Sara Di Carlo,[c] Giuseppe S. Sica,[c] Antonio Di Sabatino,[b] Giovanni Monteleone[a]

[a]Department of Systems Medicine, University of Rome 'TOR VERGATA', Rome, Italy [b]Department of Internal Medicine, San Matteo Hospital Foundation, University of Pavia, Pavia, Italy [c]Department of Surgery, University 'TOR VERGATA' of Rome, Rome, Italy

Corresponding author: Giovanni Monteleone, MD PhD, Department of Systems Medicine, University of Rome 'TOR VERGATA', Via Montpellier, 1, 00133 Rome, Italy. Tel.: +39.06.72596158: fax: +39.06.72596391; email: Gi.Monteleone@Med.uniroma2.it

Abstract

Background and Aim: The mechanisms underlying the formation of intestinal fibrostrictures [FS] in Crohn's disease [CD] are not fully understood, but activation of fibroblasts and excessive collagen deposition are supposed to contribute to the development of FS. Here we investigated whether interleukin-34 [IL-34], a cytokine that is over-produced in CD, regulates collagen production by gut fibroblasts

Methods: IL-34 and its receptor macrophage colony-stimulating factor receptor 1 [M-CSFR-1] were evaluated in inflammatory [I], FS CD, and control [CTR] ileal mucosal samples by real-time polymerase chain reaction [RT-PCR], western blotting, and immunohistochemistry. IL-34 and M-CSFR-1 expression was evaluated in normal and FS CD fibroblasts. Control fibroblasts were stimulated with IL-34 in the presence or absence of a MAP kinase p38 inhibitor, and FS CD fibroblasts were cultured with a specific IL-34 antisense oligonucleotide, and collagen production was evaluated by RT-PCR, western blotting, and Sircol assay. The effect of IL-34 on the wound healing capacity of fibroblasts was evaluated by scratch test.

Results: We showed enhanced M-CSFR-1 and IL-34 RNA and protein expression in FS CD mucosal samples as compared with ICD and CTR samples. Immunohistochemical analysis showed that stromal cells were positive for M-CSFR-1 and IL-34. Enhanced M-CSFR-1 and IL-34 RNA and protein expression was seen in FS CD fibroblasts as compared with CTR. Stimulation of control fibroblasts with IL-34 enhanced COL1A1 and COL3A1 expression and secretion of collagen through a p38 MAP kinase-dependent mechanism, and wound healing. IL-34 knockdown in FS CD fibroblasts was associated with reduced collagen production and wound repair.

Conclusions: Data indicate a prominent role of IL-34 in the control of intestinal fibrogenesis.

Key Words: IL-34; intestinal fibrosis; MAP kinase p38

1. Introduction

Inflammatory bowel diseases [IBD], mainly consisting of Crohn's disease [CD] and ulcerative colitis [UC], are chronic relapsing and remitting diseases resulting in uncontrolled inflammation of the gastrointestinal tract.[1] The clinical course of IBD is characterised by phases of remission and phases of active disease with diarrhoea, abdominal

pain, faecal blood and mucus, and weight loss. Patients can also develop local complications and/or extraintestinal manifestations.[2] One of the most common complications in IBD is intestinal fibrosis; it is typically seen in ileal CD, but it can be also found in colonic CD and UC.[3,4] Fibrostrictures [FS] occur in more than one-third of CD patients within 10 years of disease onset, and require endoscopic balloon dilatation or intestinal resection in more than two-thirds of patients at least once during their lifetime.[5,6] Secondary strictures appear at the surgical anastomosis, and more than two-thirds of patients will require an additional operation for the new stricture during the course of their life.[7,8] The pathogenesis of FS is not yet fully understood, even though it has been assumed that such complications are the result of chronic activity of inflammatory cells which stimulate excessive deposition of extracellular matrix [ECM] proteins by fibroblasts.[9,10] In the late stages of the disease, fibroblasts can secrete further profibrogenic cytokines [e.g., TGF-β, interleukin [IL]-1β, and IL-6], thereby increasing ECM deposition and perpetuating the fibrotic process.[4,11–13] Consistently, compounds blocking either profibrotic cytokines or intracellular signals triggered by such cytokines have been employed with success in the prevention or cure of mice with colitis-induced intestinal fibrosis.[14–18] However, there is no specific drug that can prevent or block intestinal fibrosis in CD, and drugs used to limit the ongoing mucosal inflammation [e.g., immunesuppressors, biologics] are not able to prevent stricture formation. The identification of molecules involved in the pathogenesis of intestinal fibrosis could facilitate the development of ant-fibrotic drugs.

IL-34 was originally identified as a regulator of myeloid cell survival and function, and was subsequently shown to have pleiotropic functions regulating a multitude of processes.[19] IL-34 is constitutively expressed in human small intestine and colon, and its production is markedly elevated in inflamed gut of patients with IBD, where the cytokine is supposed to play a role in amplifying inflammatory pathways.[20–22] Indeed, IL-34 stimulates intestinal mucosal mononuclear cells to produce inflammatory cytokines and gut epithelial cells to secrete chemoattractants.[20,22] IL-34 biological activity is mediated by interaction with the homodimeric macrophage colony-stimulating factor [M-CSF] receptor 1 [M-CSFR-1; also known as CFS-1R or FMS].[19] A second uncovered receptor of IL-34, PTP-ζ, is primarily expressed on neural progenitors and glial cells.[23] Macrophages and to a lesser extent non-immune cells, such as smooth muscle cells, neurons, trophoblasts, and osteoclasts, express M-CSFR-1.[24–28] More recent studies have shown that IL-34 stimulates the migration and proliferation of synovial fibroblasts isolated from rheumatoid arthritis patients and the production of inflammatory cytokines by lung fibroblasts, raising the possibility that fibroblasts are another cellular target of IL-34 in vivo.[29,30] Therefore, we hypothesised that IL-34 can be involved in intestinal fibrogenesis in CD. We here investigated the expression of IL-34 in CD FS and examined whether IL-34 is a regulator of collagen synthesis by gut fibroblasts.

2. Methods

2.1. Patients and samples

Surgical specimens were taken from 10 patients with inflammatory CD [I CD] undergoing surgery for a chronic active disease poorly responsive to medical treatment, and from 27 patients with FS CD undergoing surgery for such a complication at the Tor Vergata University Hospital [Rome, Italy]. Additional ileal controls [CTR] were mucosal specimens taken from macroscopically and microscopically unaffected areas of 27 patients undergoing surgery for colon cancer. Each patient who took part in the study gave written informed consent, and the study protocol was approved by the local ethics committees [Tor Vergata University Hospital, Rome, no. 231/19].

2.2. Isolation and culture of intestinal fibroblasts

All the reagents were purchased from Sigma-Aldrich [Milan, Italy] unless otherwise specified. Intestinal fibroblasts were isolated from FS CD specimens and ileal control mucosal samples, as described elsewhere.[31,32] Fibroblasts were maintained in 75 cm^3 plastic flasks and incubated at 37°C in a humidified atmosphere, with 5% CO_2 in D-MEM containing high glucose with ultra glutamine and supplemented with 10% fetal bovine serum [FBS], 1% of penicillin [100 U/ml], streptomycin [100 µg/ml], and 1% of non-essential amino acids [all from Lonza, Verviers, Belgium] and used between passages 3 and 8. To determine whether IL-34 regulates collagen production, 5 x 10^4 normal fibroblasts were plated into each well of a 12-well plate, left to adhere for 24 h, then starved for 6 h, and finally either left unstimulated or stimulated with increasing doses of recombinant human IL-34 [25–100 ng/ml, R&D Systems, Minneapolis, MN]. After 6–48 h, cells and cell-free supernatants were harvested. Cells were used for protein and gene expression analysis, and cell-free supernatants were analysed for the content of collagen.

To examine the molecular mechanisms by which IL-34 controls collagen production, serum-starved normal fibroblasts were stimulated with recombinant human IL-34 [50 ng/mL], TNF-α [20 ng/ml, R&D Systems], or IL-6 [50 ng/ml, R&D Systems] for 30 min, then lysed, and total extracts were analysed for the content of both phosphorylated and total p38 mitogen-activated protein [MAP] kinase by western blotting. To test the specificity of SB202190, an inhibitor of p38 MAP kinase, FS CD fibroblasts were plated into each well of a 12-well plate, left to adhere for 24 h, and then transfected with SB202190 [10 µM, EMD Millipore Corporation, MA, USA] or dimethyl sulphoxide [DMSO; vehicle] for 24 h. At the end of culture, cells were used to analyse ERK1/2 and p38 MAP kinase phosphorylation by western blotting. In parallel, to evaluate the role of p38 in IL-34-induced collagen production, normal fibroblasts were pre-incubated with SB202190 for 1 h and then stimulated or not with IL-34 [50 ng/ml] for 48 h. At the end of culture, cells were used for protein expression analysis by western blotting and cell-free supernatants were analysed for collagen content. In additional experiments, 5 x 10^4 FS CD fibroblasts were plated into each well of a 12-well plate, left to adhere for 24 h, and then either left untreated or transfected with a specific IL-34 antisense oligonucleotide [IL-34 AS] or sense oligonucleotide [sense] [both used at 200 nM, Integrated DNA Technologies, Leuven, Belgium] for 24–48 h using Opti-MEM medium and Lipofectamine 3000 reagent according to the manufacturer's instructions [both from Life Technologies, Milan, Italy]. The efficiency of the transfection was determined by western blotting. Cells were used for protein expression analysis by western blotting, and cell death analysis and cell-free supernatants were analysed for collagen content.

2.3. Real-time polymerase chain reaction

A constant amount of RNA [0.5 µg/sample] was retro-transcribed into complementary DNA [cDNA], and then 1 µl of cDNA/sample was amplified using the following conditions: denaturation 1 min at 95°C; annealing 30 s at 58°C for M-CSFR-1, matrix metalloproteinases [MMP]-1, MMP-2, MMP-3, MMP-9, connective growth factor; at 60°C for IL-34, COL1A1, COL3A1, and β-Actin; followed by 30 s of extension at 72°C. Primer sequences were as follows: IL-34: forward, 5′- ACAGGAGCCGACTTCAGTAC -3' and

reverse, 5′- ACCAAGACCCACAGATACCG -3’; M-CSFR-1: forward, 5′- CTGCTCAACTTTCTGCGAAG -3’ and reverse, 5′- CTCA TCTCCACATAGGTGTC -3’; COL1A1 5′-GGACACAGAGGTTTC AGTGG-3′, and reverse, 5′-GGTGACTTTGGAGACACAGG-3’, COL3A1 5′-GGAGAATGTTGTGCAGTTTGC-3′, and reverse, 5′-CGTTTGACGTGTTGTAAGAGG-3’; MMP-1: forward, 5′-ACC TGGAGGAAATCTTGCTC-3′ and reverse, 5′-TCAGTAGAATGGG AGAGTCC-3′; MMP-2: forward, 5′-CCTGTTTGTGCTGAAGG ACA-3′ and reverse, 5′- GTACTTGCCATCCTTCTCAA-3′; MMP-3: forward, 5′-GGACCTGGAAATGTTTTGGC-3′ and reverse, 5′-TTGGCTGAGTGAAAGAGACC-3′; MMP-9: forward, 5′-GTCGAAATCTCTGGGGCCTG-3′ and reverse, 5′-AAACCG GTCGTCGGTGTCGT-3′, connective growth factor: forward, 5′-TC CGTACTCCCAAAATCTCC-3’ and reverse, 5′-AGGCACAGGTCT TGATGAAC-3’; β-actin: forward, 5’-AAGATGACCCAGATCATGT TTGAGACC-3’ and reverse, 5’-AGCCAGTCCAGACGCAGGAT-3’. mRNA expression was calculated relative to the housekeeping β-actin gene on the base of the ΔΔCt algorithm. Fibronectin and tissue factor were evaluated using commercial TaqMan probes [Applied Biosystems, Foster City, CA]. RNA expression was calculated relative to the housekeeping β-actin gene on the base of the ΔΔCt algorithm.

2.4. Total protein extraction and western blotting

Fibroblasts and human colonic samples were lysed on ice in buffer containing 10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.2 mM EGTA, and 0.5% Nonidet P40 supplemented with 1 mM dithiothreitol, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na3VO4, and 1 mM NaF. Lysates were clarified by centrifugation at 4°C for 30 min and separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and membranes were then incubated with the following antibodies: mouse anti human IL-34 [1:1000 Abcam Cambridge, UK]; rabbit anti-human M-CSFR-1 [1:500 Novus Biological Southpark Way, USA]; rabbit anti-human COL1A1 [final dilution 1:1000, Novus Biological, Italy]; rabbit anti-human COL3A1 [final dilution 1:1000, Novus Biological]; rabbit anti-human p-p38 [1:1000 EMD Millipore Corporation]; mouse anti-human p-ERK1/2 [final dilution 1:5000], followed by horseradish peroxidase–conjugated secondary IgG monoclonal antibodies [all used at final dilution 1:20000, Dako, Milan, Italy]. The reaction was detected with a sensitive enhanced chemiluminescence kit [Pierce, Rockford, IL]. After the analysis, blots were stripped and incubated with the following internal loading control: mouse anti-human β-Actin [final dilution 1:5000 Sigma-Aldrich]; mouse anti-human total p38 [final diluition 1:500, Santa Cruz Biotechnology, Inc., TX, USA]; and mouse anti-human total ERK1/2 [final dilution 1:5000 Sigma-Aldrich]. Computer-assisted scanning densitometry [Image-Lab 5.2.1, Bio-Rad Laboratories, Milan, Italy] was used to analyse the intensity of the immunoreactive bands.

2.5. Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded ileal sections of ileal CTR, I CD patients and FS CD patients. The sections were deparaffinised and dehydrated through xylene and ethanol, and the antigen retrieval was performed in Tris EDTA citrate buffer [pH 7.8] in a thermostatic bath at 98°C [Dako] for 30 min. Immunohistochemical staining was performed using mouse monoclonal antibody directed against human IL-34 [final dilution 1:50000, Abcam] and a rabbit monoclonal antibody directed against human M-CSFR-1 [final concentration 1:200, Novus Biological], incubated at room temperature [RT] for 1 h ,followed by biotin-free HRP-polymer detection technology with 3,3’diaminobenzidine [DAB] as a chromogen [MACH 4 Universal HRP-Polymer Kit, Biocare Medical]. The sections were counterstained with haematoxylin, dehydrated, and mounted. Isotype control IgG-stained sections were prepared under identical immunohistochemical conditions as described above, replacing the primary antibody with a purified mouse normal IgG control antibody [R&D Systems]. The IL-34 and M-CSFR-1-positive cells were counted in at least six fields per section using IAS 2000 System [Delta Sistemi, Rome, Italy], and data were expressed as number of cells for high power field [hpf].

2.6. Immunofluorescence

Normal and FS CD fibroblasts were plated onto chamber slides [10^4 cells/well] in complete medium. The day after, cells were gently washed with phosphate-buffered saline [PBS] and fixed in 4% paraformaldehyde for 10 min at 4°C. Chamber slides were washed three times with PBS and treated with 0.1% Triton X-100 for 20 min at RT. Blocking procedure was performed with a 10% normal goat serum in PBS containing 1% of bovine serum albumin [BSA] for 1 h at RT. Slides were then incubated overnight at 4°C with mouse anti-human Vimentin [final dilution 1:100, ThermoFisher Scientific], rabbit anti-human α-SMA [final dilution 1:50, ThermoFisher Scientific], or rabbit anti-human CD90 [final dilution 1:100, ThermoFisher Scientific]. After washing three times with PBS, slides were incubated for 1 h at RT with specific secondary antibodies coupled with Alexa Fluor Dyes [final dilution 1:2000; ThermoFisher Scientific]. Coverslips were mounted on glass slides using ProLong Gold antifade reagent with DAPI [ThermoFisher Scientific] to counterstain the DNA. Samples were analysed with a Leica DMI 4000 B fluorescence microscope [Leica, Wetzlar, Germany].

2.7. Wound healing scratch assay

Normal and FS CD fibroblasts were grown in 12-well plates to ~100% confluence in complete medium. Then, medium was removed, cells were rinsed with PBS, and the monolayer was artificially wounded by scratching across each well with a 200-μl pipette tip [“pseudo” wound approximately 1 mm diameter]. The wells were washed with PBS to remove debris, and then fresh medium containing 0.05% BSA was added. Normal fibroblasts were either left unstimulated or stimulated with IL-34 [50 ng/ml] or fibroblast-growth factor [FGF]–β [20 ng/ml Peprotech EC, London, UK] for 72 h, whereas FS CD fibroblasts were transfected with IL-34 AS or sense for 48 h. Images were taken at Time 0, and after 24, 48, 72 h, and the “pseudo” wound area was measured by Image-Lab software [Bio-Rad Laboratories]. The wound healing ability of fibroblasts at the specified time points was expressed as percentage of “pseudo” wound area respect to that at Time 0.

2.8. Analysis of cell death

To score cell death, FS fibroblasts, left untreated or transfected with either IL-34 AS or sense oligonucleotide and cultured for 48 h, were washed in PBS, stained with FITC–annexin V [AV, 1:100 final dilution, Immunotools, Friesoyte, Germany] according to the manufacturer’s instructions, and incubated with 5 mg/ml PI [Life Technologies] for 20 min at 4°C. The fluorescence was measured by flow cytometry using FL-1 and FL-2 channels of a FACSVerse [BD Biosciences] flow cytometer. Viable cells were considered as AV-/PI-cells, apoptotic cells as AV+/PI-cells, and secondary necrotic cells

were characterised by AV+/PI + staining. Data are expressed as percentage of cell death.

2.9. Sircol assay

Total collagen was measured in fibroblast supernatants by Sircol Collagen Assay Kit in accordance with the manufacturer's instructions [Biocolor, Belfast, UK].

2.10. Statistical analysis

Differences between groups were compared using the Student's t test and the Mann–Whitney U test. All the analyses were performed using Graph-Pad 6 software.

3. Results

3.1. M-CSFR-1 is over-expressed by Crohn's disease fibrostrictures

To investigate whether M-CSFR-1 expression is increased in CD, RNA transcripts for M-CSFR-1 were evaluated in ileal inflammatory [I] and FS specimens of CD patients and CTR by real-time PCR. M-CSFR-1 RNA transcripts were increased in I CD patients as compared with CTR [Figure 1A]. FS CD samples exhibited a more pronounced expression of M-CSFR-1 RNA as compared with I CD and ileal CTR samples [Figure 1A]. Analysis of total proteins extracted from additional samples of the same patients and controls by western blotting confirmed the above results [Figure 1B].

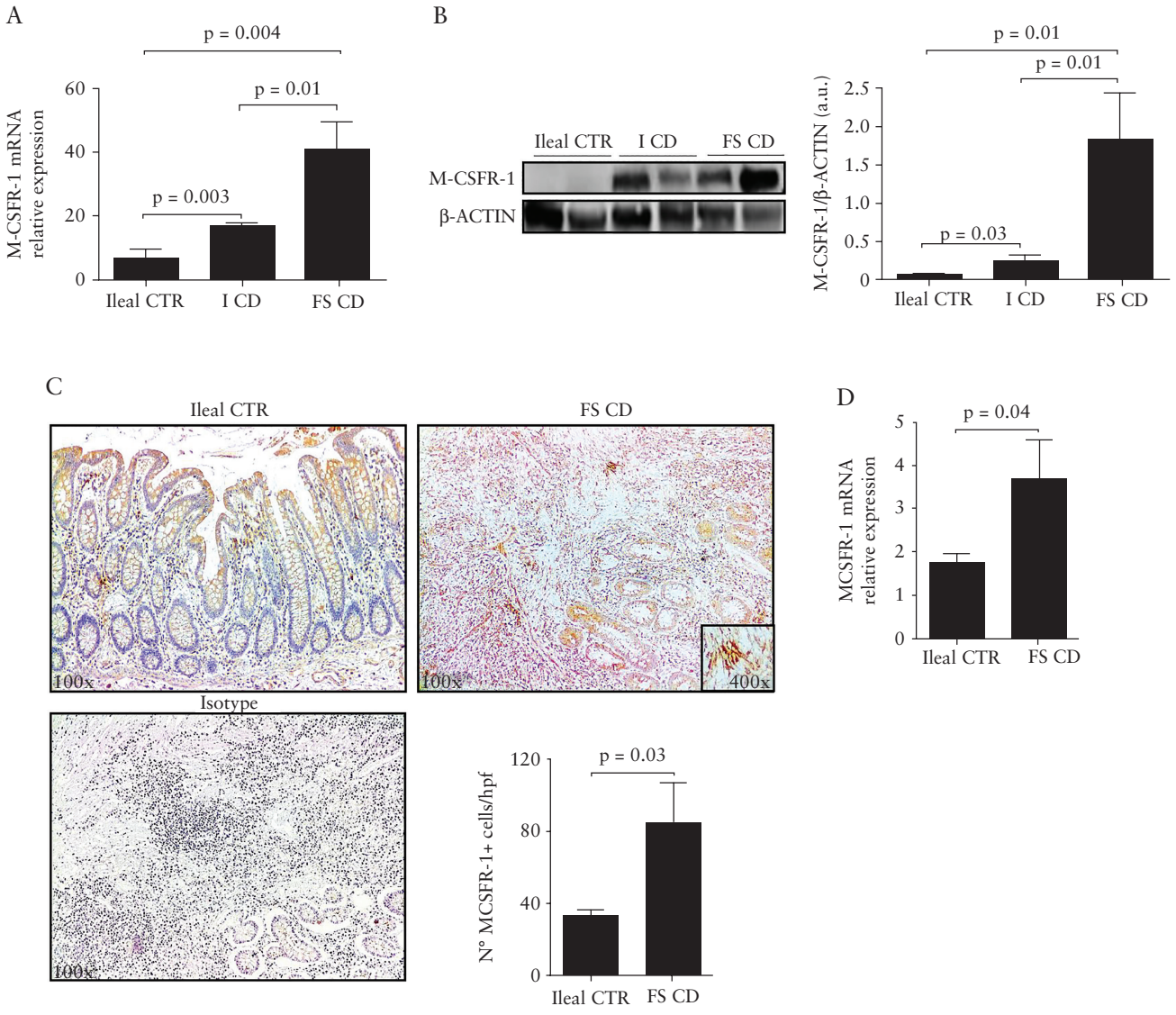

Figure 1. Macrophage colony-stimulating factor-1 receptor [M-CSFR-1] RNA and protein expression is increased in fibro-stricturing [FS] Crohn's disease [CD]. A. M-CSFR-1 RNA expression was evaluated in ileal biopsies taken from five normal ileal controls [Ileal CTR], five patients with ileal inflammatory CD [I CD], and five patients with FS CD by real-time PCR, and levels were normalised to β-Actin. Data are expressed as mean ± SEM of all samples. B. Representative western blots showing M-CSFR-1 and β-Actin in total proteins extracted from two ileal CTR, two patients with ileal I CD, and two patients with FS CD. Right panel shows the quantitative analysis of M-CSFR-1/β-Actin ratio. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all mucosal samples taken from five ileal CTR, five patients with ileal I CD, and five patients with FS CD. C. Representative photomicrographs [100x original magnification] of M-CSFR-1-stained paraffin-embedded sections of surgical intestinal samples taken from one ileal CTR and one patient with FS CD. Isotype control antibody-stained FS CD sections are also shown. Inserts show higher magnification [400x] images. Right panel shows the number of M-CSFR-1-positive cells for high power field [hpf] in ileal sections taken from three ileal CTR and three patients with FS CD. D. M-CSFR-1 RNA expressions were evaluated in fibroblasts isolated from intestinal specimens of five ileal CTR and five patients with FS CD by real-time PCR, and levels were normalised to β-actin. Data are expressed as mean ± SEM of all samples. PCR, polymerase chain reaction; SEM, standard error of the mean.

Immunohistochemical analysis showed enhanced expression of M-CSFR-1 in FS CD and showed that stromal cells were positive for the receptor [Figure 1C]. Next we isolated stromal cells from normal and FS CD specimens, and analysed M-CSFR-1 RNA by real-time PCR. Immunofluorescence analysis of such cells revealed positivity for typical markers of myofibroblasts,[33,34] including vimentin, α-SMA, and CD90 [Suppl. Fig. 1, available as Supplementary data at *ECCO-JCC* online]. Enhanced M-CSFR-1 RNA expression was seen in fibroblasts isolated from ileal FS samples of CD patients as compared with control fibroblasts [Figure 1D].

3.2. IL-34 stimulates Crohn's disease fibroblasts to produce collagen

The demonstration that intestinal fibroblasts express M-CSFR-1, and that the content of this receptor is increased in FS CD samples, prompted us to investigate whether IL-34 regulates collagen production in the gut. Stimulation of control intestinal fibroblasts with increasing doses of IL-34 [25–100 ng/ml] significantly enhanced COL1A1 and COL3A1 RNA transcripts [Figure 2A, B]. Since the maximum induction of COL1A1 and COL3A1 expression were observed when cells were stimulated with 50 ng/ml IL-34, this dose was selected for the subsequent experiments. No significant change in various MMPs and profibrotic factors, such as fibronectin, tissue factor, and connective tissue growth factor, was seen in normal fibroblasts following stimulation with IL-34 [Suppl. Fig. 2, available as Supplementary data at *ECCO-JCC* online].

Stimulation of control intestinal fibroblasts with IL-34 increased COL1A1 and COL3A1 protein expression [Figure 2C]. Moreover, quantification of soluble forms of collagen in the supernatants of fibroblast cultures stimulated with IL-34 confirmed the positive effect of the cytokine on collagen synthesis [Figure 2D].

3.3. IL-34 enhances collagen production via p38 MAP kinase-dependent pathway

Next, we explored the basic mechanism by which IL-34 regulates collagen synthesis. Since we recently showed that IL-34 activates p38 MAP kinase in epithelial cells,[22,35] a signalling pathway that controls intestinal fibroblast function,[36] the subsequent studies were carried out to examine whether the effect of IL-34 on collagen synthesis was dependent on p38 MAP kinase. To this end, control intestinal fibroblasts were either left unstimulated or stimulated with IL-34, or TNF-α, or IL-6, which were used as positive inducers of MAP kinases. IL-34 enhanced phosphorylation of p38 MAP kinase [Figure 3A], and pre-incubation of cells with a specific inhibitor of p38 [Suppl. Fig. 3, available as Supplementary data at *ECCO-JCC* online] abrogated the IL-34-induced collagen production [Figure 3B, C].

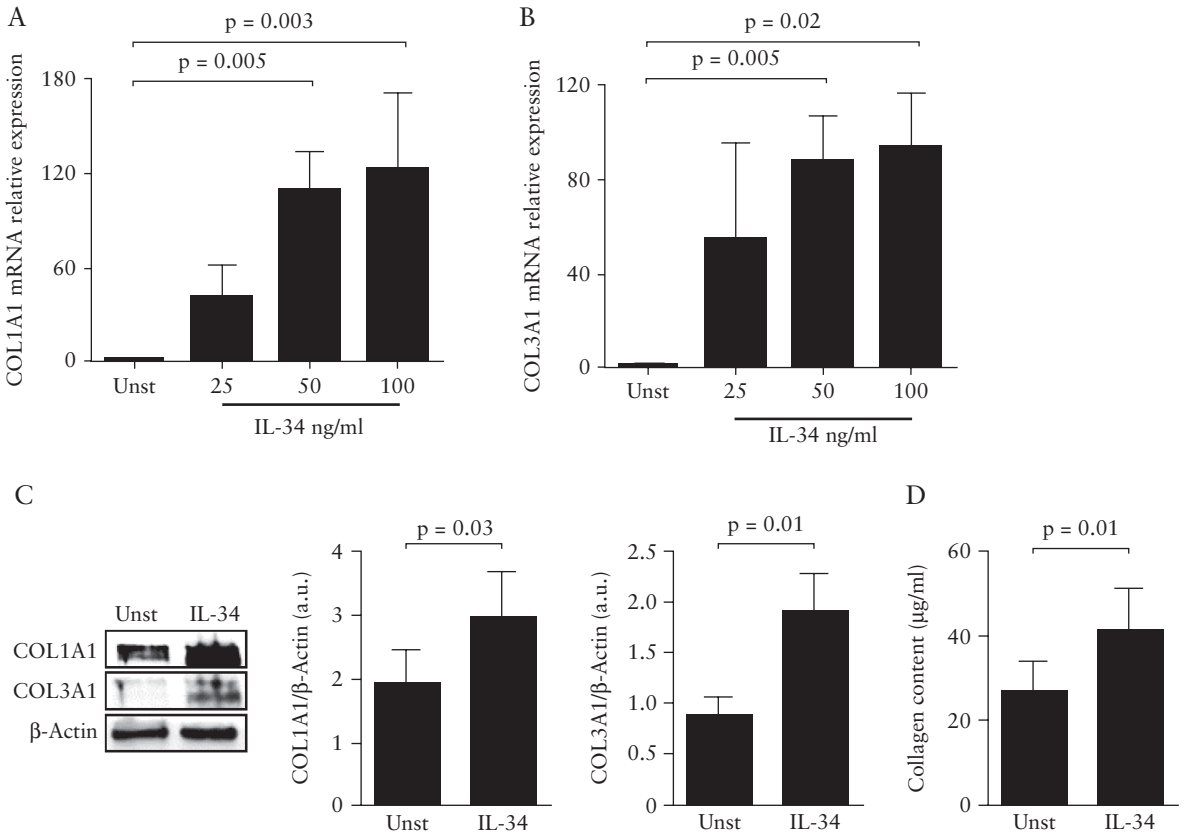

Figure 2. IL-34 enhances collagen production by normal fibroblasts. A-B. Serum-starved normal fibroblasts were stimulated with or without [Unst] increasing doses of recombinant human IL-34 [25–100 ng/ml] for 6 h, COL1A1 [A] and COL3A1 [B] RNA transcripts were analysed by real-time PCR, and levels were normalised to β-actin. Data [A-B] are expressed as mean ± SEM of four experiments. C. Normal fibroblasts were treated with or without [Unst] recombinant human IL-34 [50 ng/ml] for 48 h, and COL1A1, COL3A1, and β-actin were analysed by western blotting. One of four independent experiments is shown. Right panels show the quantitative analysis of COL1A1/β-actin ratio and COL3A1/β-actin ratio in normal fibroblast protein extracts as measured by densitometry scanning of western blots. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all experiments. D. Total content of collagen [μg/ml] in supernatants of normal fibroblasts, treated as described in C for 48 h, was analysed by a colourimetric assay. Data indicate mean ± SEM of four independent experiments. PCR, polymerase chain reaction; SEM, standard error of the mean.

3.4. IL-34 is over-produced in Crohn's disease fibrostrictures

IL-34 is produced by many cell types, including macrophages, endothelial cells, fibroblasts, neurons, and epithelial cells.[20,22,37,38] Therefore, we next investigated whether IL-34 is over-produced in CD FS samples and we examined its cell source. To this end, RNA transcripts for IL-34 were evaluated in intestinal specimens taken from I CD, FS CD, and controls. IL-34 RNA transcripts were increased in I CD patients as compared with controls [Figure 4A]. IL-34 RNA expression was more pronounced in FS CD samples as compared with control and I CD samples [Figure 4A]. Western blotting of total proteins extracted from paired mucosal samples and immunohistochemistry confirmed over-expression of IL-34 in FS CD and showed that, in FS CD, stromal cells expressed IL-34 [Figure 4B-C]. In line with the above data, enhanced IL-34 RNA and protein expression was seen in fibroblasts isolated from ileal samples of FS CD patients as compared with control fibroblasts [Figure 4D, E].

3.5. IL-34 knockdown in Crohn's disease fibroblasts associates with reduced production of collagen

We assessed the effect of IL-34 inhibition on collagen synthesis in CD fibroblasts isolated from FS specimens. Knockdown of IL-34 with a specific antisense oligonucleotide reduced both COL1A1 and COL3A1 protein synthesis as well as secretion of collagen in the culture supernatants [Figure 5A, B], without affecting cell survival [Figure 5C]. Knockdown of IL-34 reduced also phosphorylation of p38 MAP kinase [Suppl. Fig. 4, available as Supplementary data at *ECCO-JCC* online].

3.6. IL-34 increases wound healing ability of intestinal fibroblasts

In a final set of experiments, we examined the effect of IL-34 on the wound healing ability of fibroblasts using the wound healing scratch assay. IL-34 increased the wound healing ability of control fibroblasts and induced "pseudo" wound repair after 72 h, similar to that seen with FGF-β, which was used as a positive control[39] [Suppl.

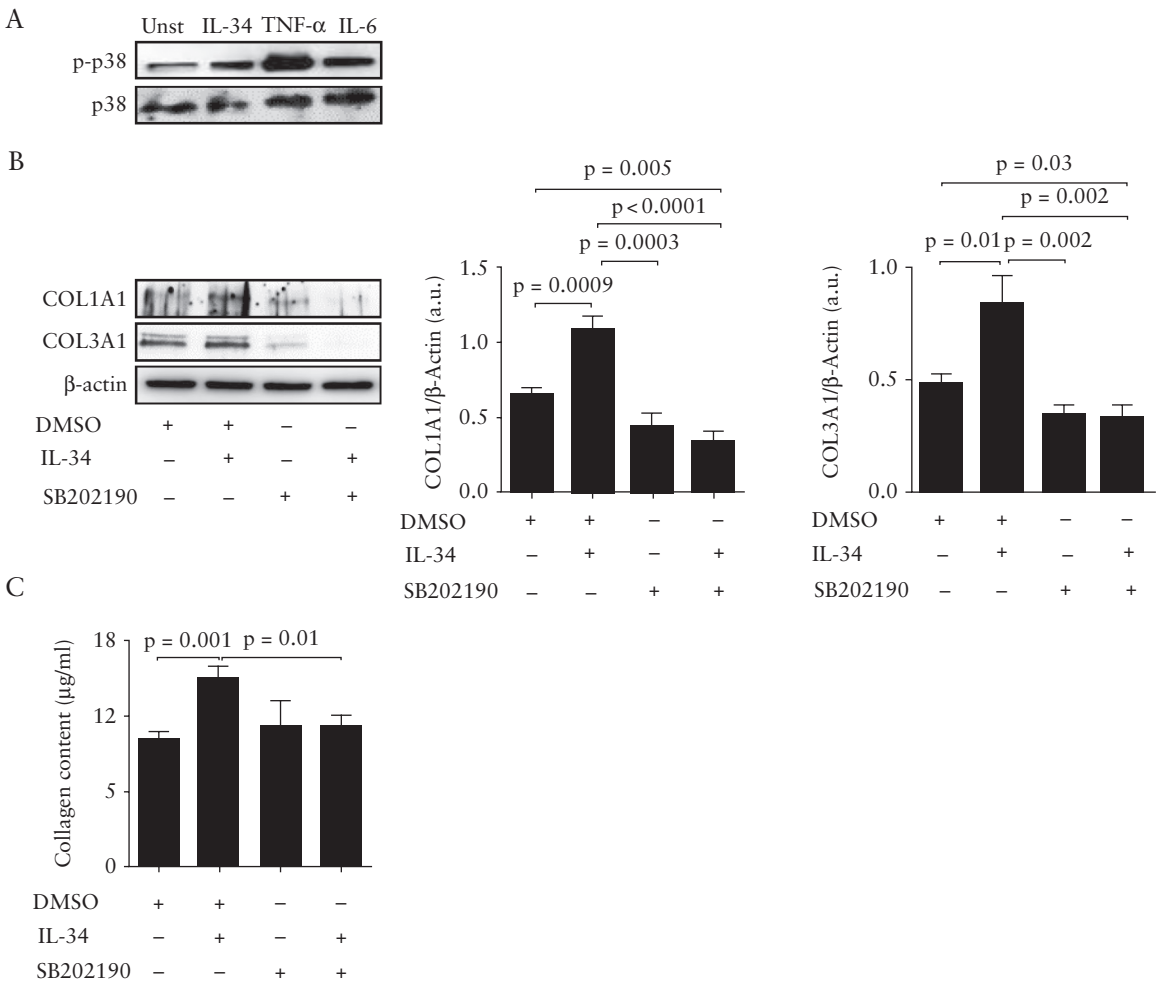

Figure 3. IL-34 induces collagen production through a p38-MAP kinase-dependent mechanism. A. Serum-starved normal fibroblasts were either left unstimulated [Unst] or stimulated with IL-34 [50 ng/ml], TNF-α [20 ng/ml], IL-6 [50 ng/ml] for 30 min. Phosphorylated and total forms of the p38 MAP Kinase were evaluated by western blotting. One of three independent experiments is shown. B. Serum-starved normal fibroblasts were pre-incubated with specific inhibitor of p38 [SB202190] or with dimethyl sulphoxide [DMSO, vehicle] for 1 h, and then stimulated with IL-34 [50 ng/ml] for further 48 h. COL1A1, COL3A1, and β-actin were evaluated by western blotting. One of five independent experiments is shown. Right panels show the quantitative analysis of COL1A1/β-actin ratio and COL3A1/β-actin ratio in normal fibroblast protein extracts as measured by densitometry scanning of western blots. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all experiments. C. Total content of collagen [μg/ml] in supernatants of normal fibroblasts treated as in B was analysed by a colourimetric assay. Data indicate mean ± SEM of five independent experiments. SEM, standard error of the mean.

Fig. 5A, available as Supplementary data at *ECCO-JCC* online]. Consistently, knockdown of IL-34 reduced the wound healing ability of FS CD fibroblasts [Suppl. Fig. 5B].

4. Discussion

The pathogenesis of CD FS is not fully understood, but circumstantial evidence suggests that many cytokines produced by both immune cells and non-immune cells induce fibroblasts to produce collagen and other mediators of the fibrogenetic process.[3] In the present work, we assessed the expression and role of IL-34 in CD FS. IL-34, a cytokine mainly involved in the survival and function of macrophages,[19] is produced in excess in inflamed IBD tissue, and functional studies have shown that IL-34 can trigger various pathways that amplify the ongoing mucosal inflammation.[20,22] Studies in other systems have also shown that IL-34 is involved in the control of fibroblasts, as it can stimulate such cells to make several inflammatory molecules.[30,38,40] However, there are no data about the influence that IL-34 exerts on intestinal fibrosis. Here, we initially showed that M-CSFR-1 expression was enhanced in inflamed intestine of patients with CD as compared with normal controls, and this occurred at both RNA and protein level. When CD patients were stratified taking into account their phenotype, M-CSFR-1 was overexpressed in vivo in strictured CD gut compared with non-strictured CD and control gut. Immunohistochemical analysis documented marked positivity of M-CSFR-1 in the stromal compartment of FS CD samples.

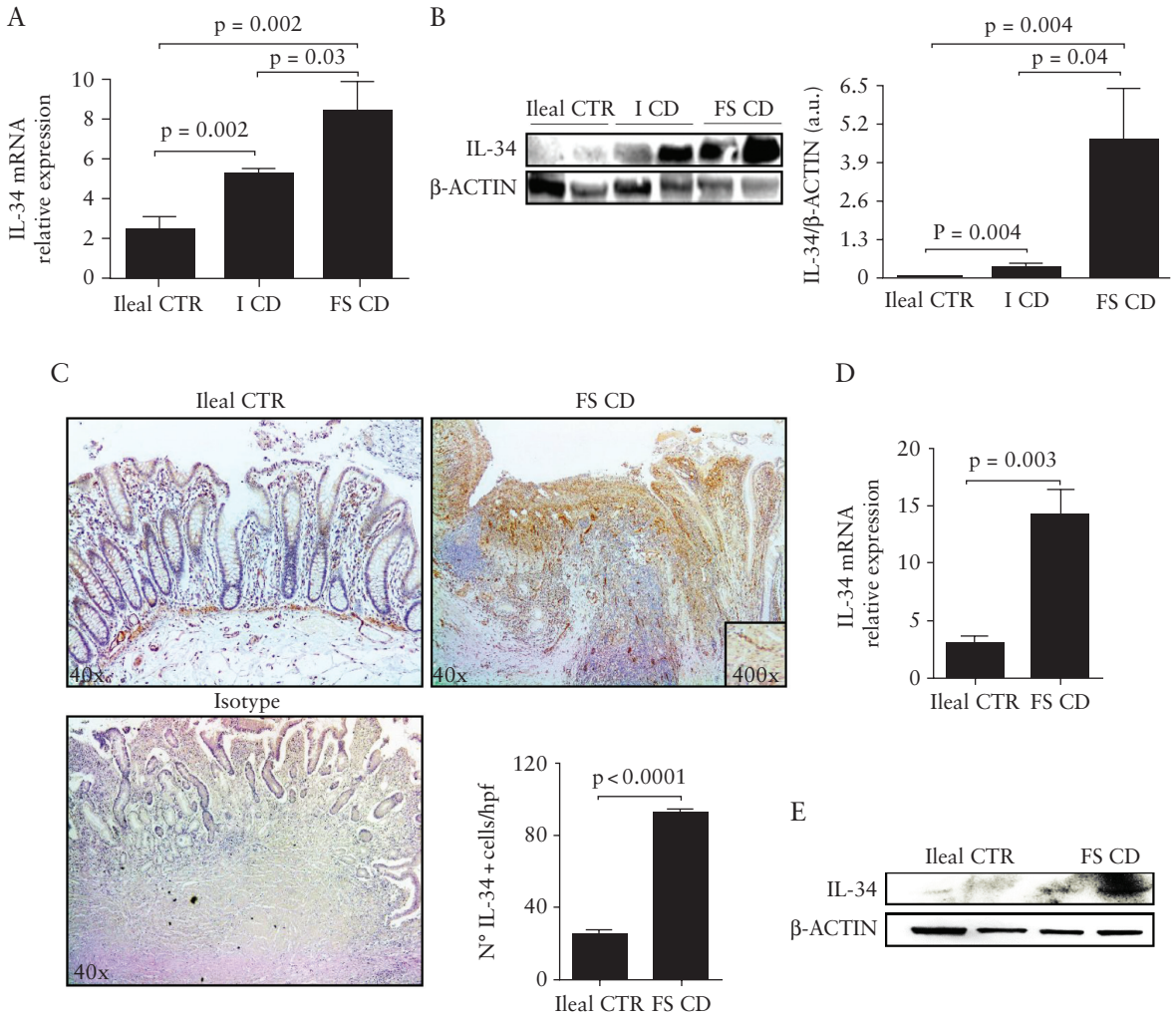

Figure 4. IL-34 RNA and protein expression is increased in FS CD tissue. A. IL-34 RNA expression was evaluated in ileal biopsies taken from five normal controls [CTR], five patients with I CD, and five patients with FS CD by real-time PCR, and levels were normalised to β-actin. Data are expressed as mean ± SEM of all samples. B. Representative western blots showing IL-34 and β-actin in total proteins extracted from two ileal CTR, two patients with ileal I CD, and two patients with FS CD. Right panel shows the quantitative analysis of IL-34/β-actin ratio. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all mucosal samples taken from five ileal CTR, five patients with ileal I CD, and five patients with FS CD. C. Representative photomicrographs [40x original magnification] of IL-34-stained paraffin-embedded sections of surgical intestinal samples taken from one normal ileal CTR and one patient with FS CD. IL-34-positive cells are seen in the stromal compartment of patients with FS CD. Isotype control antibody-stained FS CD sections are also shown. Inserts show higher magnification [400x] images. Right panel shows the number of IL-34-positive cells for high power field [hpf] in ileal sections taken from three ileal CTR and three patients with FS CD. D. IL-34 RNA expressions was evaluated in fibroblasts isolated from intestinal specimens of five ileal CTR and five patients with FS CD by real-time PCR, and levels were normalised to β-actin. Data are expressed as mean ± SEM of all samples. E. Representative western blots showing IL-34 and β-actin in total proteins extracted from fibroblasts isolated from two ileal CTR and two patients with FS CD. FS, fibrostricturing; I CD, ileal Crohn's disease; PCR, polymerase chain reaction; SEM, standard error of the mean.

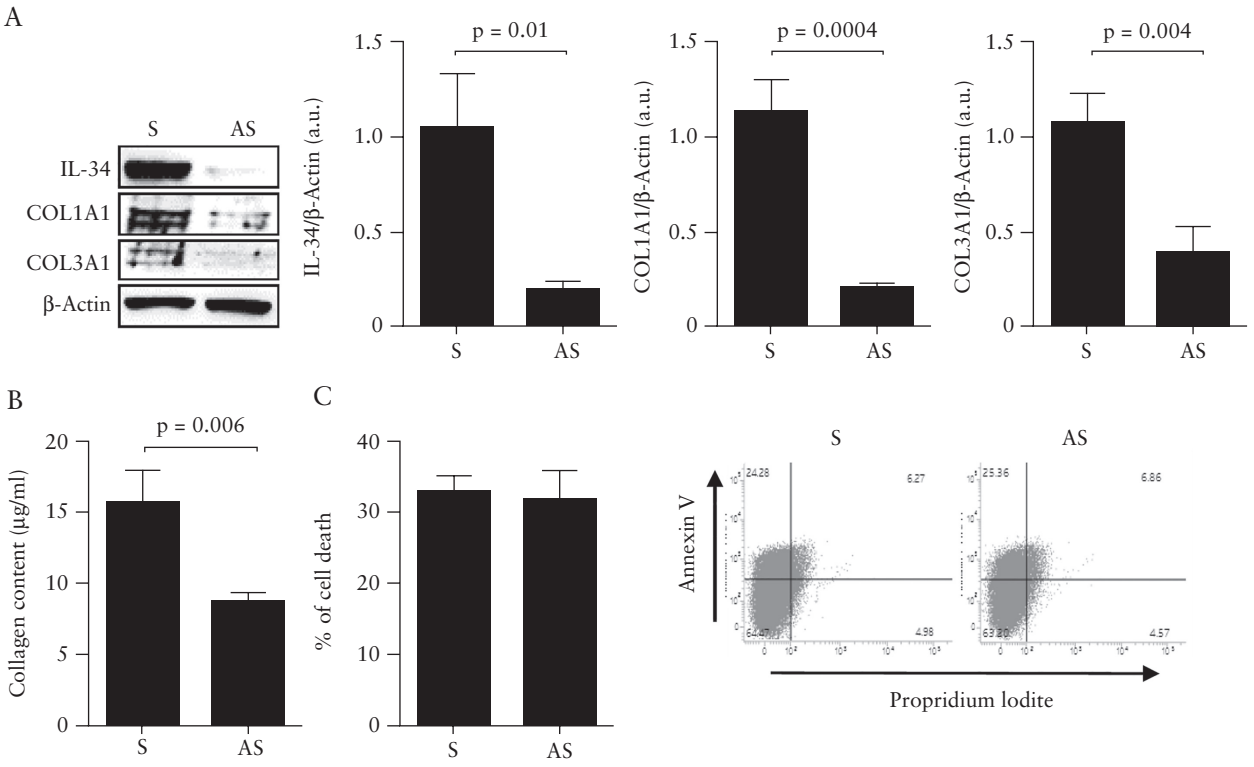

Figure 5. Knockdown of IL-34 reduces collagen production in FS CD fibroblasts. A. FS CD fibroblasts were transfected with either control sense oligonucleotide or IL-34 antisense oligonucleotide [IL-34AS] for 24 h. IL-34, COL1A1, COL3A1, and β-actin were evaluated by western blotting. One of five independent experiments is shown. Right panel shows the quantitative analysis of IL-34/β-actin, COL1A1/β-actin and COL3A1/β-actin ratio in FS CD fibroblast protein extracts as measured by densitometry scanning of western blots. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all experiments. B. Total content of collagen [µg/ml] in supernatants of FS CD fibroblasts, treated as in A for 48 h, was analysed by a colourimetric assay. Data indicate mean ± SEM of five independent experiments. C. FS CD fibroblasts were treated as described above for 48 h. The percentage of cell death was assessed by flow cytometry analysis and expressed as percentage of annexin V [AV] and/or propidium iodide [PI]-positive cells. Data are expressed as mean ± SEM of four independent experiments. Right insets show representative dot-plots indicating the percentages of AV and/or PI-positive cells treated with control sense oligonucleotide or IL-34 antisense oligonucleotide. FS, fibrostricturing; CD, Crohn's disease; SEM, standard error of the mean.

Consistently, M-CSFR-1 expression was increased in fibroblasts isolated from FS CD samples as compared with control cells. Immunofluorescence analysis showed that such cells expressed typical markers of myofibroblasts, such as vimentin, α-SMA, and CD90.[33,34] Taken together, these findings suggest that intestinal fibroblasts/myofibroblasts can be a potential target of IL-34 in CD. To further address this issue, we evaluated the profibrogenic effect of IL-34 in normal intestinal fibroblasts. Stimulation of such cells with the cytokine increased RNA and protein expression of both COL1A1 and COL3A1, thus resulting in enhanced collagen secretion. The effect of IL-34 on collagen expression does not reflect a general activation of fibroblasts, as no inducing effect of IL-34 on other pro-fibrotic markers and various MMPs was documented. Analysis of the intracellular pathways underlying the inducing effect of IL-34 on collagen showed that activation of p38 MAP kinase was essential, because pharmacological inhibition of p38 MAP kinase with a commercial compound [i.e., SB202190] abrogated the IL-34-driven collagen synthesis. We are confident about these results, as activation of p38 MAP kinase by IL-34 has been described in other cell systems and here we show that SB202190 selectively and specifically inhibits p38 MAP kinase when used at 10 µM final concentration. These data are in line with findings of previous studies showing a key role for p38 MAP kinase in controlling collagen production and fibrosis in other systems.[41,42]

The IL-34-mediated positive regulation of collagen production in the intestine was then supported by studies with fibroblasts isolated from FS CD samples, which showed that knockdown of IL-34 with a specific AS was accompanied by a significant reduction of collagen RNA and protein, as well as decreased wound healing ability of such cells.

Data emerging from recent studies support the involvement of IL-34 in other fibrotic disorders.[38,43] For instance, Preisser and colleagues showed that chronically hepatitis C virus [HCV]-infected patients with high fibrosis stages had higher serum levels of IL-34 than HCV-infected patients with lower fibrosis stages and than healthy subjects.[38] Moreover, it was shown that IL-34 promoted the acquisition of profibrotic properties by liver macrophages, because IL-34 treated macrophages induced type I collagen synthesis by hepatic stellate cells.[38] Serum IL-34 levels were also found to be increased in patients with diffuse cutaneous systemic sclerosis [SSc] as compared with patients with limited cutaneous SSc and healthy controls, and SSc patients with increased serum IL-34 levels had more often interstitial lung disease than those with normal levels.[43] Moreover, in SSc patients, serum IL-34 levels positively correlated with fibrotic scores on chest computed tomography.[43] In non-alcoholic fatty liver disease, IL-34 increased with the progression of fibrosis and was considered an independent marker for liver fibrosis.[44]

In conclusion, our study shows that IL-34 is highly produced in fibrotic gut of CD patients, and suggests a key role for this cytokine in positively regulating collagen production by intestinal fibroblasts.

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Conflict of Interest

GM has served as an advisory board member for Abbvie. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

EF performed experiments, analysed data, wrote manuscript; VD; FL, ADG, DDF. AC, AO performed experiments; PG, SDC, GS, ADS contributed reagents; GM designed experiments and wrote the manuscript.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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